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Steroids and Opioid Receptors

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The genomic mode of action is believed to represent the predominant effect of a steroid hormone. Recently, however, rapidly manifesting, non-genomic effects have also been observed. These are mediated mostly by allosteric interaction of a steroid with heterologous target structures such as membrane receptors, a prototype example being the $GABA_A$. Here we describe our studies considering two interdependent questions: (1) do steroids also interact with opioid receptors in brain? Twenty different steroids, i.e. estrogens, androgens, glucocorticoids, mineralocorticoids, gestagens and a cardiac glycoside were tested with respect to their ability to compete for *in vitro* binding to rat brain membranes of ^{3}H-ligands specific for δ, μ and κ opioid receptors, respectively. Among all classes of steroids, only the estrogens were effective, all others were 20 to 100 times less effective or ineffective. The rank order among the estrogens was diethylstilbestrol > 17α-estradiol > 17α-ethinyl-estradiol > estriol > estrone > 17β-estradiol. Next potent to estrogens (although far less) were—on average as a group—glucocorticoids, followed by mineralocorticoids, androgens, gestagens and digoxin. This global as well as within-group rank order was, with rare exceptions, qualitatively equal irrespective of which radioligand was used, yet displayed the various radioligands different sensitivities with respect of being inhibited by steroids (irrespective of kind), i.e. in the order [^{3}H]naloxone $\geqslant$ [^{3}H]DAGO $\geqslant$ [^{3}H]DADL > [^{3}H]DPDP $\gg$ [^{3}H]etorphine. The IC_{50} of diethylstilbestrol for displacing [^{3}H]DAGO was $\approx 30\,\mu M$ and that of 17β-estradiol was $\approx 200\,\mu M$. (2) What are the concentrations of the major steroid hormones in the brain's extracellular fluid? We have analyzed in 56 matched (i.e. simultaneously withdrawn) peripheral serum and cerebrospinal fluid (CSF) samples (from endocrinologically grossly normal patients) the concentrations of the unconjugated steroid hormones testosterone, androstendione, dehydroepiandrosterone (DHEA), progesterone and cortisol (all being more or less lipophilic) as well as those of their hydrophilic counterparts, i.e. DHEA-sulfate, or their hydrophilic binding proteins, i.e. sex hormone binding globulin, corticosterone binding globulin, and albumin. Total (i.e. free plus protein-bound) CSF levels of all these steroids were found to be in the 0.02–2 nM range except for cortisol ($\approx$20–50 nM), thus 3 to 4 orders of magnitude lower than the IC_{50} of estrogens for [^{3}H]DAGO (see above). These total CSF values were quite similar to the reported and calculated free serum levels of these steroids and no difference existed between those of patients with intact or with disturbed (abnormally leaky) blood–brain barrier function. Thus *in vitro* demonstrated low affinity (micromolar) interactions of steroids with neuronal membrane-bound receptors should be considered in view of the above found or expected concentrations of steroids in the interneuronal fluid. Whether *neurosteroids* can *in vivo* reach intrabrain concentrations of neuromodulatory efficacy remains to be seen; certain steroidal drugs, unlike peripheral steroid hormones, may do so.

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INTRODUCTION

Steroid hormones are known to act as allosteric modulators of their cognate receptors. The receptors thereby acquire the competence to act as nuclear transcription regulating factors on steroid-sensitive a dependent genes. This property includes various features such as translocation into and/or retention in the cell nucleus, attachment by their DNA binding domain (DBD, containing the double zinc finger motif) to a steroid responsive element (SRE) located in the 5′ upstream promoter region of a steroid-sensitive/dependent gene, and interaction with various other constitutive and cell-specific transcription factors. Steroid receptors can thus be considered as *trans*-acting factors whose *cis* elements are SREs. This in an hourly fashion

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("delayed") occurring and genomic mode of action is believed to represent the predominant effect of a steroid hormone, whether being an androgen, estrogen, gestagen, glucocorticoid or mineralocorticoid, or a vitamin D3-derivative. Thyroid hormones as well as retinoic acid display a similar mode of action in accordance with the fact that their receptors display sequence and architectural similarity with steroid receptors (for review, see [1]).

Recent interest focussed on the influence of gonadal and adrenal steroid hormones on nerve cells as rather novel target cells for such peripheral hormones [2, 3], including among others brain opioid receptors [4]. With some surprise rapidly manifesting, non-genomic effects have also recently been observed in nerve cells. Membrane receptors and ion channels appear to be a major class of such heterologous target structures for certain steroids, the $GABA_A$ receptor being the prototype example [5, 6] (for reviews, see [7, 8]). Such direct interaction of steroids has often been shown by modulation of radioligand binding to the respective membrane receptor.

We therefore became interested into two questions:

(1) Do steroids also interact with opioid receptors in brain? Can this be shown *in vitro* by inhibition or enhancement of [^{3}H]opioid ligand binding to rat brain membranes when increasing concentrations of different unlabeled steroids are present? If so, is there a specificity as to the class or type of steroid as well as the opioid receptor subtype? Opioid receptors occur in at least 3 established subtypes, δ (vas deferens), μ (morphine) and κ (ketocyclazocine) based on *in vivo* pharmacology [9], differential ligand binding [10], tissue as well as within-brain regional distribution [11], and other criteria of specificity. Therefore, subtype-specific [^{3}H]-opioid ligands were used as well as 20 different steroids representing members of each of the major steroid classes (estrogens, androgens, gestagens, mineralocorticoids, glucocorticoids and for comparison a cardiac glycoside).

(2) What are the concentrations of peripheral (blood-borne) steroid hormones in the brain's extracellular fluid? In particular, how do these relate to reported and in this study determined *in vitro* potencies of steroids? We therefore measured in 56 matched (i.e. simultaneously withdrawn) serum and cerebrospinal fluid (CSF) samples the concentrations of the major steroid hormones, i.e. testosterone, androstendione, dehydroepiandrosterone (DHEA), progesterone and cortisol (all being more or less lipophilic, as determined by octanol/saline partitioning) and compared them with the serum and CSF concentrations of their hydrophilic counterparts, i.e. DHEA-sulfate, or their hydrophilic binding proteins, i.e. sex hormone binding globulin (SHBG), corticosterone binding globulin (CBG), and albumin. For each parameter, individual and averaged CSF/serum ratios were determined.

We found that (1) under the experimental conditions chosen, opioid receptors, unlike $GABA_A$ and others receptors, are unlikely to be targets for steroid hormones, except, perhaps, for estrogens if present at micromolar concentrations, and (2) that all classical (i.e. peripheral) steroid hormones occur in CSF at nanomolar concentrations, i.e. below 5 nM except for cortisol (<50 nM).

MATERIALS AND METHODS

Inhibition of [^{3}H]opioid ligand binding to rat brain membranes

We incubated rat brain membranes with either 1 of 5 different ^{3}H-ligands specific for the δ, μ, and κ subclass of opioid receptors, respectively, in the presence of increasing concentrations of 1 of 20 different unlabeled steroids belonging to each of the major steroid classes (estrogens, androgens, gestagens, mineralo-, glucocorticoids and, for comparison, a cardiac glycoside). For each steroid, a complete dose–response curve was generated in order to determine its inhibitory concentration 50% (IC_{50}) with respect to each subclass of opioid receptors.

Ligands. Steroids were purchased from Sigma. Stock solutions (100 μM) were prepared in abs. EtOH, from which working solutions were prepared by dilution in Tris.Cl, pH 7.4 buffer.

Tritiated ligands, DADL ([D-Ala^2,D-Leu^5]-enkephalin), DPDP ([D-Penicillamine^2,D-Penicillamine^5]-enkephalin), DAGO ([D-Ala^2,MePhe^4,Gly-ol^5]-enkephalin), and naloxone, were purchased from New England Nuclear (Boston, MA), etorphine was from Amersham Labs (Amersham, Bucks., England). The specific radioactivities (Ci/mmol) were 36.6 for [^{3}H]DADL-acetate, 43.0 for [^{3}H]DPDP, 47.7 for [^{3}H]DAGO, 44.5 for [^{3}H]naloxone, and 50 for [^{3}H]etorphine, respectively. The original ethanolic solutions of these substances were kept at -20°C. Before use, appropriate volumes were directly diluted in assay Tris–BSA buffer. Because of the light sensitivity of [^{3}H]naloxone, all experiments were performed under dimmed yellow light.

Preparation of rat brain membranes (RBM). Ten brains were usually processed in one batch. Two-month-old male Sprague–Dawley rats were anesthesized by CO_2-inhalation for 2 min and then decapitated. The brains were dissected from pituitary and cerebellum, rinsed several times with ice-cold 50 mM Tris.Cl buffer, blotted dry, and the tissue wet weight (tww) recorded. Nine volumes of this buffer were added and brains were homogenized with a Polytron (Brinkman, Westbury, NY) twice at a setting of 6.0 for 15 s. The slurry was centrifuged at 30,000g for 10 min in a Sorvall SA-600 rotor. The pellet was

resuspended with a hand-held glass–Teflon homogenizer, washed 3 times in the same volume of buffer with intermittent centrifugations at 30,000*g* for 10 min each. After resuspension of the final pellet in the same volume of buffer, protein content was measured by the Bradford method using a modified Coomassie Blue G-250 based protein reagent (Pierce Chem. Comp., Rockford, IL) with bovine serum albumin (BSA) (Sigma) as standard. Optical density was read at 595 nm. The preparation was adjusted to a protein concentration of 2 mg/ml, corresponding to ≈100 mg tww/ml. The protease inhibitors bestatin and bacitracin (Sigma) were then added to give a concentration of 100 nmol and 200 μg, respectively, per ml RBM suspension. This RBM suspension was kept on ice until use in binding assay.

Binding assays. All studies were performed using freshly prepared RBM, 50 mM Tris.Cl buffer, pH 7.4 (pH adjusted at room temperature), duplicate or triplicate determinations and a total incubation volume of 1 ml. The binding protocol was as follows: to glass assay tubes (12 × 75 mm), chilled in ice water, were added in sequence: 0.1 ml of unlabeled steroid (or buffer for B_0 tubes), 0.1 ml of labeled opioid ligand (at a final concentration of 0.5 nM), 0.1 ml of 10 mg/ml BSA in Tris.Cl buffer and 0.2 ml Tris.Cl buffer. After brief vortex mixing, 0.5 ml of the ice-cold RBM suspension was added and the tubes were incubated for 30 min in a shaking water bath at 37°C. After chilling for 10 min, tubes were rapidly filtered through Whatman GF/B glass fiber filter paper using high negative pressure in a M-48 Multi-Probe Cell Harvester (Brandel, Gaithersburg, MD). Each tube and respective filter disc was rinsed 3 times with 3 ml ice-cold Tris.Cl buffer. The discs, retaining the RBM-bound labeled ligand, were transferred to glass mini counting vials, incubated for a minimum of 12 h with 5 ml Ready-Solv HP cocktail (Beckman, Mountainside, NJ) to ensure their complete dissolution, and subsequently counted in a Packard Tricarb spectrometer for 5 min at ≈40% efficiency. Using external standardization and automatic quench correction, all results were expressed as dpm. In each assay, non-specific binding (NSB = maximum inhibition) was assessed in tubes containing 10 μM homologous unlabeled opioid ligand or naloxone. In a typical experiment 5214 ± 250 dpm (mean ± SD, replicates within experiment) [^{3}H]DADL were bound per filter disc, 2206 ± 254 dpm [^{3}H]DPDP, 7682 ± 213 dpm [^{3}H]DAGO, 6727 ± 359 dpm [^{3}H]naloxone, 5200 ± 467 dpm [^{3}H]etorphine. NSB was between 0.4 and 1.6% of total labeled ligand added.

Data analysis. Results were expressed as specific binding (B_s), i.e. the difference between total binding (B_t) of radioactivity and NSB. Complete dose–response curves were generated from the B(ound) vs log[steroid] (M) inhibition plots. Where applicable, i.e. when inhibition ranged to levels below 50% ($B/B_0 < 0.5$), IC_{50} values were determined using the 4-parameter logistic function as implemented in the computer program ALLFIT [12].

Measurement of steroid and steroid binding globulin levels in serum and CSF

Selection of patients and assessment of blood–CSF barrier (BCB) function. Remnants of serum and CSF samples, kept deep frozen at −20°C, were analyzed. These had been previously obtained from neurological patients as part of their routine neurological examination (including CSF chemistry, BCB function and X-ray myelography) and already analyzed with respect to total protein, albumin and IgG using conventional clinical chemistry photo- and nephelometric methods (Beckman Immunochemistry System, Beckman Instruments Inc., San Ramon, CA). Serum and CSF samples had been withdrawn by venipuncture and lumbar puncture, respectively, whereby the initial 0.2 ml of CSF were always discarded to avoid possible contamination with blood.

A commonly used criterion for distinguishing an intact BCB from a disturbed BCB is the application of an upper normal limit of 45 mg/100 ml for the total protein concentration in CSF and a value of 0.007 for the CSF/serum concentration ratio of albumin [13]. Hence, we could choose from the set of samples pairs of CSF and serum that belonged to patients prediagnosed to have either intact BCB ($n = 36$) or disturbed BCB ($n = 20$). Selection within each group was done in a random manner, except for the fact that we had to allow for sufficient sample volume. Within both groups, none of the 56 patients had any overt endocrinological symptoms or diseases.

Reagents. The following radioactive steroids were from New England Nuclear (Dreieich, Germany): [^{3}H]-androstendione (sp. act. 85 Ci/mmol), [^{3}H]cortisol (98.5 Ci/mmol), [^{3}H]DHEA-sulfate ammonium salt (19.6 Ci/mmol), [^{3}H]progesterone (115 Ci/mmol), [^{3}H]5α-dihydrostestosterone (5α-DHT, 50.6 Ci/mmol). [^{3}H]Testosterone (80 Ci/mmol) was from Amersham. The following highly specific rabbit antisera were used: anti-androstendione (K_a 3.3×10^9 $l.M^{-1}$, sensitivity 5 pg/tube, BioMakor, Kiryat Weizmann, Rehovot, Israel), anti-cortisol (K_a 6.8×10^9 $l.M^{-1}$, sensitivity 10 pg/tube, BioMakor), anti-DHEA-sulfate (with 100% crossreactivity to DHEA, Wien Labs., Succasunna, NJ), anti-progesterone (K_a 5.0×10^{10} $l.M^{-1}$, sensitivity 5 pg/tube, BioMakor), anti-testosterone (K_a 1.4×10^{10} $l.M^{-1}$, sensitivity 5 pg/tube, BioMakor).

Determination of SHBG and CBG. Determination of SHBG was performed as described previously in detail [14, 15]. Briefly, 0.25 ml aliquots of CSF or of 1:50 diluted plasma, respectively [diluted with assay buffer: 0.05 M phosphate, pH 7.4, supplemented with 1% (w/v) of bovine serum albumin] were titrated with increasing doses (in 0.05 ml) of [^{3}H]5α-DHT (0.2–2 nM) in the absence and presence, respectively, of 200 nM unlabeled 5α-DHT (for determination of total and non-specific binding, respectively). Total

incubation volume was 0.35 ml. After 120 min of incubation at 4°C, the mixtures were treated for 10 min with 0.8 ml cold dextrane coated charcoal suspension [DCC: 0.5% (w/v) activated charcoal (Merck), 0.05% dextrane T-70 (Pharmacia, Uppsala, Sweden) in assay buffer] and then centrifuged at 800*g*. Supernatants (0.8 ml, containing the bound radioactivity) were emulsified in liquid scintillation cocktail and measured in a beta scintillation spectrometer. Determination of CBG was similar to that of SHBG, except that the stripped serum samples were diluted 1:400 (CSF was diluted 1:2 like in SHBG assays). As described by others [16], aliquots of 0.3 ml were assayed together with 0.1 ml [^{3}H]cortisol (0.4–4 nM) and 0.1 ml of buffer or 0.1 ml unlabeled cortisol (400 nM), respectively. After incubation for 60 min at 37°C and for 30 min in ice water, 0.5 ml cold DCC were added and samples treated in the same way as above for SHBG. All samples were stripped prior to assay from endogenous steroids by incubation with an equal volume of cold DCC for 5 min at 4°C. This step was previously found to be mandatory in those cases where excessive amounts of steroidal drugs are present [14]. Scatchard plot evaluation of specifically bound counts was performed using a previously described computer program in order to derive the binding capacity of SHBG and CBG in nmol/l serum or CSF [17]. The K_d of SHBG and CBG, respectively (as determined by Scatchard analysis), is under the conditions specified 0.2–0.4 and 0.8–1.2 nM, respectively, and thus well within the range of these radioligand concentrations used.

Radioimmunoassay (RIA). RIA of steroid was performed as described previously in detail [18]. Varying volumes of sample were extracted by organic solvent. Solvents were then dried down under a gentle stream of nitrogen gas and redissolved in varying volumes of assay-buffer (same as above for SHBG and CBG) depending on the expected steroid levels in the sample and the sensitivity of the assay. Aliquots of 0.1 ml of such extracts were incubated for 120 min at 4°C with 0.1 ml ^{3}H steroid ($\approx$50 pg/tube corresponding to $\approx$10,000 cpm at 37% efficiency) and 0.1 ml appropriately diluted antiserum. Thereafter, each RIA tube received 0.8 ml of cold DCC suspension (same as above) and was further treated as described above. For RIA of cortisol, 0.1 ml serum or CSF, respectively, were extracted with 1 ml dichlormethane of which 0.1 and 0.5 ml, respectively, were dried down and redissolved in 1 ml buffer. For RIA of testosterone, DHEA or androstendione, 0.5 ml serum or CSF were extracted with 8 ml of diethyl ether, the entire extract dried down and redissolved in 1 ml (for serum) or 0.25 ml buffer (for CSF), respectively. For RIA of progesterone, 0.25 ml serum or 1 ml CSF, respectively, were extracted with petrol ether and the dried residues redissolved in 1 ml or 0.25 ml buffer, respectively. For RIA of DHEA-sulfate, samples were measured directly without extraction, serum being 1:200 diluted with buffer, CSF being undiluted. Each RIA contained

Table 1. Inhibition of [^{3}H]opioid ligand binding to RBM

	100 μM	NALX* $\mu + \delta + ka$	DAGO* μ	DADL* δ	ETOR* ka
Estrogens	Diethylstilbestrol	0.25*	0.22	0.34	0.65
	17α-Estradiol	0.32	0.32	0.45	0.70
	17α-Ethinyl-estradiol	0.41	0.37	0.49	0.75
	Estriol	0.55	0.55	0.54	0.75
	Estrone	0.55	0.58	0.52	0.85
	17β-Estradiol	0.40	0.60	0.73	1.00
Glucocorticoids	17α-HO-Progesterone	0.88	0.96	0.86	
	11-Deoxycortisol	0.82	0.82	0.72	
	Prednisolone	1.00	0.77	0.69	0.88
	Dexamethasone	0.84	0.94	0.93	1.00
	11-Deoxycorticosterone	0.75	0.80	0.72	
Mineralocorticoid	D-Aldosterone	0.87	0.87	0.80	
Androgens	4-Androsten-3,17-dione	0.78	0.79	0.75	
	Dehydroepiandrosterone	0.85	0.99	0.78	
	Testosterone	0.87	0.86	0.70	
	5α-Androstan-17β-ol-3-one	0.95	0.99	0.73	
	Androsterone	0.91	0.80	0.79	
	Danazol	0.95	1.00	1.00	0.92
Gestagen	Progesterone	0.88	1.00	1.00	
Cardiac Glycoside	Digoxin	0.83	0.91	0.82	1.00
Estrogens		0.413**	0.440	0.512	0.783
Glucocorticoids		0.858	0.858	0.858	0.940
Mineralocorticoid		0.870	0.870	0.800	
Androgens		0.885	0.905	0.792	
Gestagen		0.880	1.000	1.000	
Cardiac Glycoside		0.830	0.910	0.820	1.000
		0.789***	0.831	0.797	0.908

*Individual B/B_0 values; **per steroid group averaged B/B_0 values; ***per opioid radioligand averaged B/B_0 values.

6 standards in $\log_2$ dilutions that covered a concentration range from 15 to 250, or 30 to 500 or 125 to 4000 pg/tube, respectively, as appropriate to the affinity of the respective antiserum.

All determinations were performed in duplicate assay tubes. Computer-assisted data evaluation and quality control of RIA was performed according to procedures described previously in detail [19]. Determinations had to be performed in several consecutive assay batches. Therefore, to account for possible drifts in assay performance, not only were two quality control samples included in each batch but, more importantly, a roughly equal number of matched CSF and serum samples from both groups of patients were analyzed concurrently.

RESULTS

Interaction of steroids with opioid receptors

Table 1 shows the results obtained with all the 20 steroids that were tested as to their ability to inhibit [^{3}H]opioid ligand binding to rat brain membranes *in vitro*. The [^{3}H]opioid ligands were NALX* (naloxone), DAGO*, DADL* and ETOR* (etorphine). Although for each steroid a complete dose–response curve was generated that covered 4 orders of magnitude, this table contains only the data pertaining to the highest concentration point ("-4" in Fig. 1), i.e. 1×10^{-4} M (100 μM). As can be seen, only estrogens were effective, i.e. produced more than 50% binding inhibition (B/B_0 values below 0.5). By comparison, all other steroids were less effective or not effective at all ($B/B_0 \approx 1.0$).

Only with the *estrogens*, the range of inhibition was such that calculation by ALLFIT of the IC_{50} was feasible. A typical example is shown in Fig. 1 (upper panel). Here it is indicated that for inhibiting binding of the μ-selective ligand [^{3}H]DAGO, diethylstilbestrol (DES) was strongest followed by 17α-estradiol (aE2L), 17α-ethinylestradiol (aEE2L), estriol (E3L), estrone (E1N), and 17β-estradiol (bE2L) being the weakest. The respective IC_{50}s, calculated by the computer program ALLFIT, are given in the inset to Fig. 1.

This rank order among the estrogens, DES $\geqslant$ aE2L $\geqslant$ aEE2 > E3L $\geqslant$ E1N $\geqslant$ bE2L, was the same whatever opioid radioligand was used, as can easily be seen by inspecting in vertical manner the (increasing) B/B_0 values given in Table 1 (which represent the "-4"-point in Fig. 1).

Further it was interesting to see which opioid radioligand was most and which least susceptible to interference in binding by steroids. Figure 1 (lower panel) shows that [^{3}H]naloxone (NALX*) was most susceptible, followed by DAGO*, DADL*, DPDP* and ETOR*, when tested against those steroids that were most active, i.e. the estrogens; aE2L, the second strongest inhibitor (Fig. 1, upper panel), being taken as an example. The ALLFIT-calculated IC_{50}s for 17α-estradiol are given in the insert to the lower panel Fig. 1. This rank order, NALX* > DAGO* > DADL* > DPDP* > ETOR* was the same whatever estrogen was used, as can easily be seen by inspecting in horizontal manner the (increasing) B/B_0 values given in Table 1.

In contrast to estrogens, the inhibitory potency of all the other steroids was too small to allow such statements as made above for estrogens. Concentration points greater than 100 μM could not be set up because of lack of solubility of steroids in water. Still, if one takes the various steroids together as groups, as shown in the lower part of Table 1, i.e. if one looks vertically at the group-averaged B/B_0 values, it appears that glucocorticoids are the second most potent inhibitors, followed by mineralocorticoids, androgens, gestagens and the cardiac glycoside digoxin. This order appears to be roughly the same for each of the various opioid radioligands used. Likewise, if one compares the averaged B/B_0 values for each of the opioid radioligands, NALX* is most susceptible and ETOR* is least susceptible, a rank order also found with

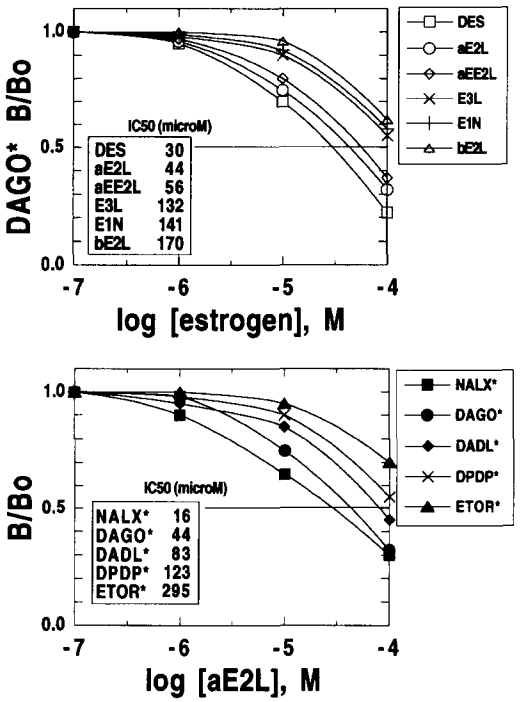

Fig. 1. Inhibition of binding of [^{3}H]DAGO to rat brain membranes by various estrogens (upper panel) and inhibition of binding of various [^{3}H]opioid ligands by 17α-estradiol (aE2L) (lower panel). Increasing concentrations as given on the abscissa) of either one of the various estrogens, diethylstilbestrol (DES), 17α-estradiol (aE2L), 17α-ethinyl-estradiol (aEE2L), estriol (E3L), estrone (E1N), 17β-estradiol (bE2L), were incubated with a constant amount of [^{3}H]DAGO (upper panel) and rat brain membranes, increasing concentrations (as given on the abscissa) of 17α-estradiol (aE2L) were incubated with a constant amount of either [^{3}H]naloxone (NALX*), or [^{3}H]DAGO, or [^{3}H]DADL, or [^{3}H]DPDP, or [^{3}H]etorphine (ETOR*) (lower panel) and rat brain membranes. Data represent mean values of bound [^{3}H]radioligand (B) expressed as a fraction of that in the absence of steroid (B_0). The insets show the respective IC_{50} values.

Table 2. Correlations and cross-correlations between CSF and serum levels

CSF levels						
DHEAS	DHEA	ADN	CORT	TEST	PROG	Serum levels
0.001	0.001	0.001	—	—	—	DHEAS
	0.001	0.001	—	—	—	DHEA
		0.001	—	—	—	ADN
			0.001	—	—	CORT
				0.001	—	TEST
					0.001	PROG

CSF levels					
alb	IgG	SHBG	CBG	DHEAS	
0.001	—	—	—	—	Albumin
	0.001	—	—	—	IgG
		0.001	—	—	SHBG
			0.001		CBG
				0.001	DHEAS

0.001, $P < 0.001$ for r (coefficient of correlation, as calculated by linear regression analysis); —, $P > 0.1$ for r, DHEA: dehydroepiandrosterone, DHEAS: DHEA-sulfate, ADN: androstendione, CORT: cortisol, TEST: testosterone, PROG: progesterone, alb: albumin, IgG: immunoglobulin G, SHBG: sex hormone binding globulin, CBG: corticosterone binding globulin.

the estrogens, only that DAGO* and DADL* were reversed.

Steroid levels in CSF

As shown in Table 2, the CSF levels of every parameter measured were always positively correlated with the respective serum levels. The higher the individual serum level, the higher the individual CSF level, indicating that all parameters measured were truly blood-borne. This correlation was independent of the size and chemical nature of the parameter, i.e. whether it was (a) a small hydrophilic molecule such as DHEA-S (left panel of Fig. 2), (b) a large hydrophilic molecule such as SHBG (middle panel), or (c) a small lipophilic parameter such as progesterone (right panel).

Furthermore, Table 2 shows the lack of cross-correlations between the parameters. This indicates that within an individual, each parameter transgresses the BCB or blood–brain barrier (BBB) with its own rate independent of the rate of any of the other parameters. The only exception was seen with the steroids androstendione (ADN), DHEA and DHEA-S, which is expected since their individual serum levels were also cross-correlated, in accordance with the fact that they all stem from the same origin, i.e. the zona reticularis of the adrenal gland, and that they are under common pituitary control, i.e. CASH (human adrenal gland cortical androgen stimulating hormone, [20]). Note the lack of cross-correlation of ADN or DHEA with CORT (cortisol), again expected because cortisol stems from the zona fasiciculata and is under the control of ACTH.

Marked differences were seen in the CSF levels of the various parameters which were in obvious dependence of their physicochemical nature. Accordingly, also the CSF/serum ratios were markedly different. As can be seen from Fig. 3 (open symbols

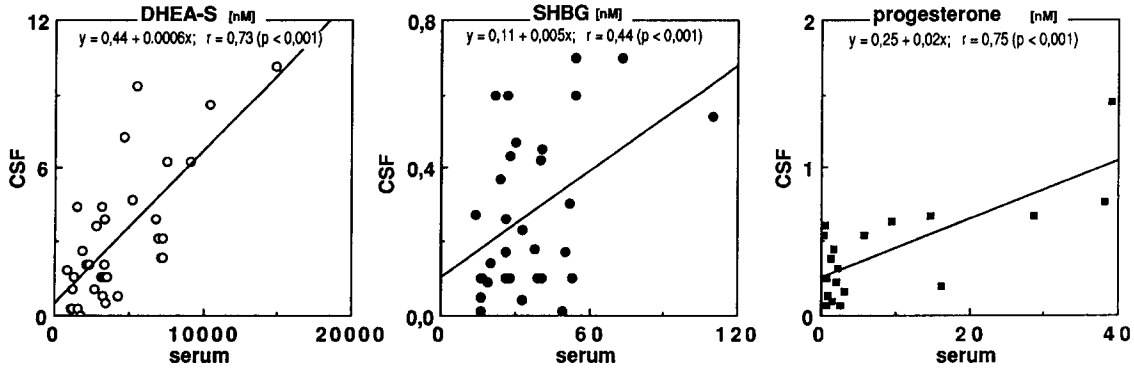

Fig. 2. Correlation and linear regression analysis of measured individual total concentrations in CSF with those in serum of DHEA-S (left panel), SHBG (middle panel), and progesterone (right panel). All values are given in nmol/l (nM) serum or CSF, respectively. Equations of the lines of regression are shown together with the coefficient of correlation and the P value ($P < 0.001$ denoting high statistical significance).

representing the averages of the 36 individuals with intact BCB/BBB), the lowest CSF/serum ratio had DHEA-S, ≈10 times higher ones had the various hydrophilic proteins, SHBG > albumin > CBG > IbG, and ≈100 times higher were those for the lipophilic steroids: ADN > PROG (progesterone) > CORT (cortisol > TEST (testosterone) > DHEA. This finding is in accordance with the concept that the BCB as well as the BBB allow lipophilic substances to permeate more readily than hydrophilic ones [21].

This conclusion is further corroborated by also including in our study 20 patients with disturbed BCB/BBB. As can be seen from the closed symbols in Fig. 3, CSF/serum ratios of hydrophilic parameters (DHEA-S and proteins) were markedly higher than those in individuals with intact BCB/BBB, whereas those of the lipophilic steroids were not different. The elevation of CSF levels of DHEA-S and proteins in patients with abnormally leaky BCB/BBB function was similar (i.e. 2–3)fold as those of albumin and corresponded with the individual degree of severity of the BCB/BBB disturbance, the latter being routinely judged by the CSF/serum ratio of albumin [13].

These differences found in the behavior between normal and disturbed BCB function clearly shows that the hydrophilic class of substances permeates the BCB/BBB only by mechanisms characterized by "restricted diffusion" (e.g. by means of carriers or transporters, or by transcytosis) while little or no such impediment is imposed on the lipophilic class of molecules [22–28].

Table 3 summarizes the averaged CSF levels of steroids as well as of SHBG, albumin and CBG found in individuals with intact BCB/BBB. For steroids, the total (i.e. free plus protein-bound) CSF levels are given. As can be seen, except for cortisol, all were in the 0.02–2 nM range. CSF levels of cortisol were ≈20–50 nM. We were unable to measure CSF levels of 17β-estradiol and aldosterone, because we estimated them to be even lower than 0.02 nM, as actually measured in a few specimen, so that too large volumes of CSF would have been required for extraction.

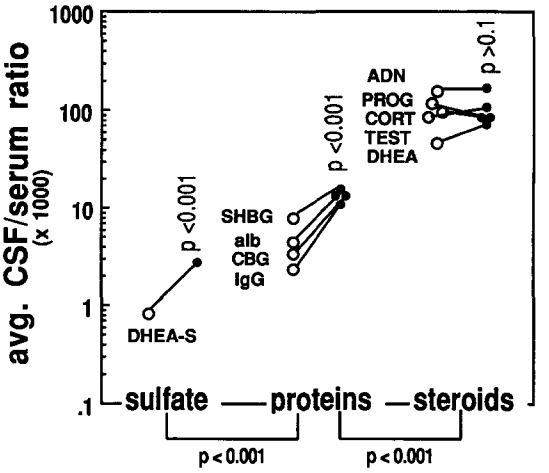

Fig. 3. Averaged CSF/serum ratios (taken × 1000) of various parameters found in 36 individuals with normal BCB/BBB (open symbols) compared with those found in 20 patients with disturbed ("leaky") BCB/BBB (closed symbols). Note that the CSF/serum ratios of each of the three groups differ significantly ($P < 0.001$, written horizontally at the bottom) when compared within the individuals with normal BCB/BBB. Note further that the CSF/serum ratios of only the hydrophilic parameters (DHEA-S and proteins), but not those of the lipophilic steroids, are significantly higher in patients with disturbed BCB/BBB when compared to those with intact BCB/BBB (*P* values written vertically).

As can be seen further from Table 3, these *total CSF levels* were quite similar to the reported [29] and calculated *free serum levels* of these steroids. This indicates that free steroids are freely diffusible through the BCB/BBB and that protein-bound steroids are not. Table 3 also shows that absolute CSF levels for SHBG and albumin are 0.34 nM and 14.9 mg/dl (≈2 mM), respectively, compared to serum levels of 43 nM (the averaged serum level for women) and 4500 mg/dl (606 mM), respectively. Thus, on average, in individuals with normal BCB only a fraction of 1/125 of serum SHBG (=CSF/serum ratio of 0.008) nd of 1/222 of serum albumin (=0.0045) is present in CSF.

Table 3. CSF chemistry of 36 patients with intact BCB

		Measured total CSF concentration[a]	Calculated free serum concentration[b]	%Unbound in plasma[c]	CSF/serum ratio[d]
Androstendione	(nM)	1.1	0.10–1.85	7.54	0.1570
Progesterone	(nM)	0.32	0.01–0.85	2.36	0.1160
Cortisol	(nM)	26.1	5.7–62	3.77	0.0990
Testosterone	(nM) female	0.20	0.01–0.30	1.30	0.0853
Testosterone	(nM) male	0.74			
DHEA	(nM)	1.67	0.10–5.00	3.93	0.0470
DHEA-sulfate	(nM)	3.02			0.0008
SHBG	(nM) female	0.34			0.0080
Albumin	(mg/100 ml)	14.9			0.0045
CBG	(nM)	0.51			0.0033

[a]Total, protein-bound + protein-unbound concentration; [b]total serum concentration converted into free concentration using the respective %unbound values given in the column to the right (range of values found in the 36 patients with intact BCB), [c]values taken from Ref. [29]; [d]total CSF/total serum concentration; from individually calculated CSF/serum ratios.

From these data one can therefore derive the following conclusion: if the presence in serum of ≈40 nM SHBG together with ≈600 nM albumin results in a calculated %unbound (= % free) value of 1.3% for testosterone (see Table 3), this value in CSF should become almost 100%, given that only 1 out of 100–200 serum SHBG and albumin molecules transgresses into the CSF compartment. This also applies for all other steroid hormones.

DISCUSSION

Besides binding with pico- to nanomolar affinities to their intracellular (cytosolic/nuclear) receptors and mediating genomic actions [1], recently evidence is being accumulating that steroids can also bind, despite considerable lower affinities, to plasma membrane structures in brain and other tissues. This has been shown by direct saturation binding using tritiated steroids [30] or by inhibition of binding of radioligands specific for a variety of receptors, ion channels and transporters or electrophysiological studies. Pertinent examples are the inhibition by progesterone of [^{3}H]quinuclidinyl benzylate binding to muscarinic receptors (estrogens being ineffective) [31], the inhibition of extraneuronal catecholamine uptake by virtually all steroids [32], the presynaptic effect of 17β-estradiol in converting striatal D_2-dopamine receptors into a low-affinity agonist binding state [33], the insulin resistance syndrome due to cortisol excess-induced diminished insulin receptor affinity or post-receptor defects [34], the potency of glucocorticoids in diminishing under certain circumstances the affinity of the tumor necrosis factor receptor on lymphocytes [35], inhibition by progesterone of binding of [^{3}H](+)*N*-allylnormetazocine [(+)SKF10,047] to haloperidol-sensitive sigma ("opioid") receptors in brain [36], binding of progesterone to the digitalis receptor [37], or membrane receptor-mediated electrophysiological effects of glucocorticoids on neurons [38].

The best studied example is certainly the $GABA_A$ receptor–chloride channel complex [5, 6], at which certain progestins not only act in an allosteric manner with nanomolar affinity but also with convincing structural specificity: e.g. 3α-HO-5α-dihydroprogesterone behaving as a barbituate-like agonist [5] and Δ5-pregnenolone-sulfate as an antagonist [39]. Our study showed that rat brain μ, δ and κ opioid receptors are, under the experimental conditions chosen, insensitive to both gestagens and all other steroids except for estrogens.

Relative to the interaction of progestins with the $GABA_A$ receptor estrogens were with respect to opioid radioligand binding inhibition ≈1000 times less potent requiring 20–200 μM concentrations to be effective. Still, they did so with some structural specificity, i.e. in a particular rank order. This, however, was largely independent of the opioid radioligand used. Vice versa, the rank order in susceptibility of different opioid radioligands to be inhibited in binding by estrogens was independent of the particular estrogen used. Thus, although the opioid radioligands chosen differed both in terms of receptor subtype specificity and in chemical structure, i.e. morphinan structure for naloxone ($\mu + \delta + \kappa$), peptidic structure for the enkephalin analogs DAGO (μ), DADL (δ) and DPDP (δ), and a non-morphinan structure for etorphine (κ).

Thus, the inhibitory activity of steroids pertained only to those that possess an aromatic ring, that is estrogens, irrespective of whether being of truly steroidal nature, such as 17α-estradiol, or belonging to the group of non-steroidal estrogens, such as diethylstilbestrol, Interestingly, the "unnatural" 17α-estradiol was 3 times more potent than the natural 17β-estradiol.

Most opioid ligands carry an aromatic ring moiety, whether belonging to the group of opioid peptides, such as Leu-enkephalin (Tyr-Gly-Gly-Phe-Leu), or to the group of opioid alkaloids, such as morphine or naloxone (Fig. 4). These ligands, however, bind/compete with nanomolar affinities to/with opioid receptors [9–11, 40, 41]. Although tyrosine alone was completely without effect in inhibiting opioid radioligand binding (data not shown), it is conceivable, that the aromatic moiety contained in opioid as well as in non-opioid substances, even when so diverse as estrogens, as shown here, or calcium channel antagonist

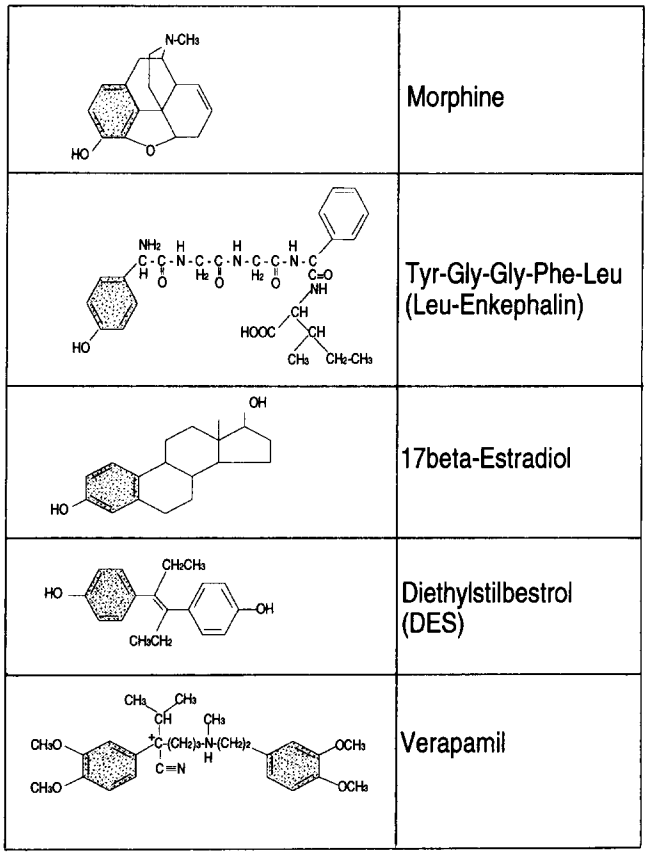

Fig. 4. Structures of opioid ligands (of morphan and peptidic structure), of estrogens (of steroidal and non-steroidal structure) and of the calcium channel blocking drug verapamil. Note the presence of an aromatic hexa carbon ring moiety (shaded) in every compound shown.

drugs such as nifedipine, verapamil or diltiazem, as shown previously [42] (Fig. 4), is the common denominator by which, with micromolar affinity, a hydrophobic (phenyl-accepting) subpocket within the opioid ligand binding domain of opioid receptors can be "probed". This value of affinity may be considered as a microscopic or partial affinity of the total affinity displayed by true opioid ligands. This subpocket appears to be present in μ, δ and κ opioid receptors, Recent cloning of the δ receptor [43] and a κ-like receptor [44] indicates that they are of partial homology, in particular with respect to membrane topology, i.e. both belong to the superfamily of 7 transmembrane helix receptors. It is noteworthy that adrenoceptors, the prototype member of this class, captures catecholamine ligands, also containing an aromatic ring, by certain, recently in site-directed mutagenesis experiments defined, amino acid residues exposed within the pocket that is formed by these 7 transmembrane helices [45]. Although it can not be excluded, it is unlikely that the compounds (steroids, calcium channel blockers) we have used here would have "probed" structures other than opioid receptors, e.g. cytochrome *P*450 enzymes, also present in brain homogenates and involved in neurotransmitter and drug metabolism (see article of Warner *et al.*, this volume), since those would have barely been labeled with the low concentrations of [^{3}H]opioid ligands employed.

The second part of the present study has re-established some important general properties of the BCB/BBB in the context of steroid endocrinology, namely that only about 1 out of 100 to 1 out of 1000 hydrophilic molecules (DHEA-S, SHBG, CBG, albumin) circulating in blood can transgress into the CSF and brain compartment whereas, among lipophilic steroid molecules, every single one can do so, as long as it is free, i.e. not bound to SHBG, CBG or albumin. Protein-bound steroids underly the same degree of restriction as their corresponding binding proteins. Although we can not rule out the possibility

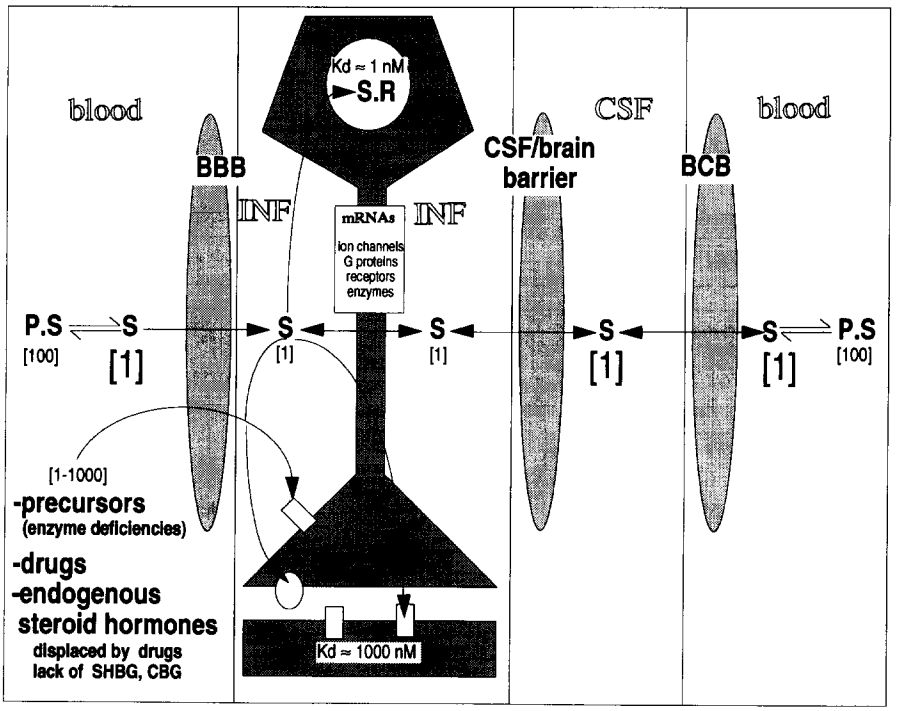

Fig. 5. Schematic diagram displaying steroid fluxes between various compartments and possible steroid-mediated neuromodulation. It is shown that in blood most steroids are protein-bound (P.S) and only a small (<1%) percentage are free (S). Numbers in square brackets denote concentrations in nM as actually measured in this study (Table 3). According to Table 3, only the free fraction of steroids appears to be able to transgress into the brain's aqueous compartments. Therefore, beyond the BCB, the concentration of a steroid is, at best, ≈1–2 nM just as is its free concentration in blood. This freely diffusible steroid would also transgress the CSF brain barrier as well as the blood–brain barrier (BBB). Hence, both in the CSF and the interneuronal fluid or even in the synaptic fluid concentrations of a particular steroid would be, at best, ≈1–2 nM (only cortisol would amount to maximally 50 nM). Given the low concentrtions of steroids within the brain tissue's extracellular fluids, high affinity ($K_d \approx 1$ nM) interaction and activation of the steroids' cognate intracellular receptors is likely which results in long-term neuromodulation by regulation of expression of a number of neuronal proteins involved in signalling (ion channels, neurotransmitter receptors, G proteins, neurotransmitter reuptake carriers, neurotransmitter generating and metabolizing enzymes etc.). Less likely than this genomic mode of action is the direct interaction of steroids (in particular when present at physiological, i.e. low nanomolar concentrations) with already existing signalling components (non-genomic steroid-mediated neuromodulation), given that *in vitro* measured potencies are generally in the micomolar range ("$K_d \approx 1000$ nM). Certain possibilities, however, are conceivable under which excessive concentrations of steroids can accumulate *in vivo* (see text in Discussion section).

that such proteins can carry along steroid into the CSF, the contribution of such transport is certainly extremely small (compare 0.51 nM as the averaged CSF value of CBG with 26.1 nM as that for total cortisol, Table 3). If protein-unbound = "free" plasma steroids diffuse freely into the CSF, they should do so into any other of the brains "inner" extracellular compartments, such as the interneuronal fluid or the synaptic fluid.

As has previously been shown for progesterone [41], CSF levels of all major steroid *hormones* (except for cortisol) as well as adrenal androgens are below 5 nM (cortisol being below 50 nM) (Table 3). Since the serum levels of all *precursors* for all steroid hormones are markedly lower than those of the respective hormones, and also in part bound to serum steroid binding globulins [29], they would also barely reach values above 5 nM. And also would do so sulfated or glucuronylated *metabolites* of steroids, despite their serum levels being 1000 times higher than the respective hormone levels, as can be seen from DHEA-S and DHEA (Table 3). Since CSF levels of peripheral (i.e. blood-borne) steroids were not higher than free serum levels, one can anticipate that nowhere in the brain's extracellular fluids steroid concentrations higher than those in CSF should be present. This concept is schematically depicted in Fig. 5. These data are thus in accordance with the "Free Hormone Theory" [46, 47] but not necessarily in contradiction to alternative theories [48].

Recently, the same authors who have described membrane-bound progesterone receptors in *Xenopus laevis* oocytes [49] also have discovered cytochrome *P*450scc activity in brain [50] and suggested the existence of brain-borne steroids ("neurosteroids") [51]. Despite rigorous quantification of their actual levels in the brain's extracellular fluids are as yet missing, it is still possible that some of those neurosteroids may locally reach higher concentrations than those 5 nM that peripheral steroids reach.

The CSF levels of all major steroid hormones, as well as steroid precursors and metabolites appeared to be 3 to 4 orders of magnitude lower than the IC_{50} value of the most potent estrogen for inhibition of [^{3}H]DAGO binding (Fig. 1). Whether this *in vitro* potency accurately reflects *in vivo* affinity is not clear since steroids may *in vivo* also interact with neuronal membrane components in a manner not readily measurable *in vitro* by inhibition of radioligand binding. Nevertheless, *in vitro* demonstrated interactions of blood-borne steroids with neuronal membrane-bound receptors or ion channels always should be considered in perspective to the above found or expectable concentrations in the *interneuronal fluid*, in particular if they were of low affinity.

High concentrations of steroids may also simply act non-specifically in an anesthetic-like fashion by changing the membrane fluidity [52–56] and thereby indirectly alter the properties of membrane-embedded receptors and ion channels [57]. Certain steroidal *drugs*, rather than natural peripheral steroid *hormones*, may, however, reach intra-brain concentrations of possible neuromodulatory potency, in particular if they are given at high doses and if they are not or not sufficiently bound to plasma SHBG or CBG and albumin. Also it is possible that due to diminished serum concentrations of SHBG, CBG and albumin, natural steroids hormones can transiently increase in the brain compartment.

Likewise, steroids become displaced from SHBG and CBG when certain steroidal drugs are administered [14]. Steroids act genomically in the CNS, both in an organizational-irreversible and in an activational-reversible manner and do so by coordinated regulation of a variety of more or less steroid-dependent or indirectly steroid-sensitive genes for neurotransmitter receptors and uptakers, neurotransmitter generating/metabolizing enzymes as well as ion channels and other structures, such as post-receptor transduction and effector molecules ("permissive regulation", [58]). Elucidation of the molecular mechanisms of steroid action in the human brain is important since reports showed induction of severe behavioral changes both by administration of steroids as well as by their withdrawal [59–61].

This study links measured steroid levels in human CSF (and serum) with their *in vitro* inhibitory potency on rat opioid receptors, two systems that are, admittedly, not directly comparable. Nevertheless, we believe that the major conclusions put forward in this study are valid, given that the neuroendocrine systems are known to be evolutionary highly conserved, be it the molecular architecture and measurable affinity of receptors (steroid and opioid [61] receptors alike) as well as steroid binding globulins, or be it the systemic total and free concentrations of steroid hormones. This study was done because neither rat CSF was available nor human brains. In the rat, most steroid levels are lower than in man [62] implying that, most likely, the CSF concentrations are also lower, which thus underscores our conclusions, namely that unphysiologically high concentrations of steroids would be required should they effectively interact with brain opioid receptors.

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